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ORIGINAL ARTICLE

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Anti-tumor drug candidate 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole induces single-strand breaks and DNA-protein cross-links in sensitive MCF-7 breast cancer cells

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Abstract *Purpose*: The fluorinated benzothiazole analogue 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203, NSC 703786) exhibits selective and potent anticancer activity, and its lysylamide prodrug (Phortress, NSC 710305) recently entered Phase I clinical trials in the United Kingdom. Only cancer cells sensitive to the anti-proliferative effects of 5F 203 deplete this drug candidate from nutrient media. 5F 203 induces cell cycle arrest, cytochrome P450 1A1 (CYP 1A1) mRNA and protein expression, and is metabolized into reactive electrophilic species that can covalently bind to DNA and form adducts in sensitive (i.e., MCF-7) but not in resistant (i.e., MDA-MB-435) breast cancer cells. *Methods*: In this present study, we investigated additional anticancer effects of 5F 203 in MCF-7 cells. In addition, we sought to determine if cells deficient in the xeroderma pigmentosum D gene, a gene critical in DNA repair, would show greater sensitivity to the cytotoxic effects of 5F 203 than those complemented with XPD. *Results*: Alkaline Elution revealed that 5F 203 induced single-strand breaks and DNA-protein cross-links in sensitive MCF-7 cells. In contrast, we detected no double-strand breaks or protein-associated strand breaks typically associated with topoisomerase I (top1) or topoisomerase II (top2) inhibition. In addition, 5F 203 was unable to trap top1- or top2-DNA cleavage complexes in MCF-7 cells. 5F 203 induced cell cycle arrest in MCF-7 cells following DNA damage after brief exposures. Cells deficient in the nucleotide excision repair xeroderma pigmentosum group D (XPD) gene displayed sensitivity to 5F 203 while cells complemented with XPD displayed resistance to 5F 203. *Conclusion*: These data suggest that the anti-cancer activity of 5F 203 depends upon targets other than top1 or top2 and on the ability of this benzothiazole to form single-strand breaks and DNA-protein cross-links in cancer cells.

Keywords Breast cancer · 5F 203 · DNA damage · Single-strand breaks · DNA-protein cross-links

Abbreviations DPCs: DNA-protein cross-links · SSBs: DNA single-strand breaks · DSBs: DNA double-strand breaks · PASBs: Protein-associated strand breaks · ISCs: Interstrand cross-links · top1: Topoisomerase I · top2: Topoisomerase II · AhR: Aryl hydrocarbon receptor · FBS: Fetal bovine serum · QRT-PCR: Quantitative real-time reverse transcription-polymerase chain reaction · DMSO: Dimethyl sulfoxide · PBS: Phosphate buffered saline · XRE: Xenobiotic response element · 5F 203: 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (NSC 703786) · DF 203: 2-(4-amino-3-methylphenyl)benzothiazole (NSC 674495)

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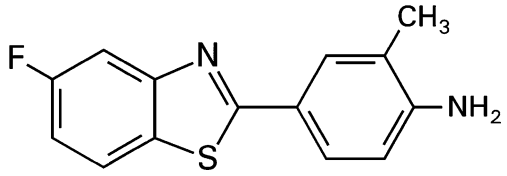

Fig. 1 Chemical structure of the anti-tumor drug candidate 5-fluoro-2-(4-amino-3-methylphenyl)benzothiazole (5F 203, NSC 703786)

Introduction

The fluorinated benzothiazole analogue 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203, NSC 703786, Fig. 1) represents a potent, selective inhibitor of cancer cell growth in vitro and in vivo [11, 15]. However, the exact mechanism by which 5F 203 displays its anticancer activity remains yet to be fully elucidated. 5F 203 was synthesized to circumvent the tendency of sensitive cancer cells to metabolize the unfluorinated benzothiazole (DF 203) into an inactive hydroxyl metabolite (6OH 203) believed to thwart its anti-tumor activity [12]. Due to its superior chemotherapeutic profile, a lysylamide prodrug of 5F 203 is currently undergoing evaluation in phase I clinical trials in the UK. Aminoflavone, a candidate agent with a similar anticancer activity profile, will soon undergo clinical evaluation in the US.

The aryl hydrocarbon receptor (AhR) pathway is involved in mediating the anticancer effects of 5F 203 and aminoflavone [20, 21, 35]. 5F 203 activates the AhR signaling pathway by inducing the translocation of AhR from the cytoplasm to the nucleus. Once in the nucleus, the AhR binds to xenobiotic response elements located in the promoter region of AhR-responsive genes and initiates gene transcription. CYP1A1, an AhR-responsive gene, has been shown to play a critical role in the anticancer activity of benzothiazoles [7, 11, 12, 20]. 5F 203 is metabolized into reactive intermediates that can bind covalently to macromolecules (e.g., DNA) within sensitive cells after inducing CYP1A1 mRNA and protein expression. 5F 203 has also been shown to induce the expression of DNA damage-responsive genes such as DNA binding protein 2 (DDB2), TNFRSF6 (CD95/FAS), CDKN1A (p21/Cip1) and p53 induced-gene 3 [23]. Recent evidence has demonstrated a correlation between cell sensitivity and the extent of DNA adduct formation in cells following 5F 203 treatment since 5F 203 readily induces DNA adduct formation in sensitive cells while only minimal DNA adduct formation occurs in resistant cells [35].

The objective of this study was to evaluate DNA damage caused by 5F 203 in sensitive MCF-7 breast cancer cells. In this investigation, we use alkaline elution analyses to fully elucidate the cellular and DNA damage responses induced by 5F 203 treatment in sensitive MCF-7 breast cancer cells. We demonstrate that 5F 203 induces single-strand breaks, DNA-protein cross-links and cell cycle arrest in sensitive MCF-7 cells. Our data suggest that sensitive cells may demonstrate susceptibility to the anticancer effects of 5F 203 because of the diminished involvement of the nucleotide excision repair (NER) pathway in the repair of DNA adducts that may form in these cells. The ability of 5F 203 to readily generate specific DNA-interactive adducts in sensitive cells provides further insight into the mechanism of anti-proliferative activity for this anticancer drug candidate.

Materials and methods

Cell culture, enzymes and chemicals

Human MCF-7 and MDA-MB-435 breast cancer cell lines were cultured in antibiotic-free, RPMI 1640 medium (Gibco-Invitrogen) containing 10% fetal bovine serum (FBS) and 2 mM glutamine. XPD and XPD-complemented fibroblasts were cultured in DMEM (Gibco-Invitrogen) with 10% heat inactivated FBS complemented with L-glutamine. Recombinant human top1 was purified from TN5 insect cells (HighFive; Invitrogen Corp., San Diego, CA, USA) using a Baculovirus construct for the NH_2 terminus-truncated human top1 cDNA as described previously [39]. Recombinant human top2α was expressed in yeast and purified as previously described [16]. DNA polymerase I (Klenow fragment) and T4 polynucleotide kinase were purchased from GIBCO/BRL. [α^{32} P]dGTP and [γ^{32} P]ATP were purchased from New England Nuclear, polyacrylamide was purchased from Bio-Rad, and etoposide (VP-16) and DMSO were purchased from Sigma. Camptothecin (CPT) was provided by the National Cancer Institute Drug Synthesis and Chemistry Branch (Rockville, MD, USA). 5F 203 was synthesized by previously described methods [12], submitted to NCI-Frederick from the University of Nottingham (Prof. Malcolm Stevens) and stored in the repository of the Developmental Therapeutics Program. Drug stock solutions of 10 mM dissolved in dimethyl sulfoxide (DMSO) were stored protected from light at −20°C until use. Before use, the stock solution was diluted to the desired concentrations in RPMI medium such that the final DMSO concentration did not exceed 0.1%.

In vitro 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

MCF-7 cells grown in 96-well plates were exposed to varying concentrations of 5F 203 for 6 h before anticancer activity was determined using a method previously published [2]. The percentage of cell growth inhibition relative to the untreated control is represented as the mean ± SD of at least three independent experiments.

Alkaline elution assay

DNA damage was detected using alkaline elution assays as described previously [10, 32]. MCF-7 and MDA-MB-435 cells were labeled with [^{3}H] thymidine (1.0 μCi/ml) for 72 h. Cells were chased overnight (16 h) with radioisotope-free medium before receiving 5F 203 treatments. The cells were harvested following specified incubations by scraping them into ice-cold HBSS.

Single-strand breaks (SSBs) and interstrand cross-links (ISCs)

Single-strand breaks and ISCs were analyzed using DNA-denaturing (pH 12.1) alkaline elution carried out under deproteinizing conditions as described previously [10].

DNA-protein cross-links (DPCs) and protein-associated strand breaks (PASBs)

DNA-protein cross-links and PASBs were analyzed using DNA-denaturing (pH 12.1) alkaline elution carried out under non-deproteinizing conditions as described previously [4, 10, 34].

Double-strand breaks (DSBs)

Double-strand breaks were analyzed using non-DNA denaturing (pH 9.6) elution carried out under deproteinizing conditions as described previously [10, 34].

DNA flow cytometric analysis

Samples were prepared for cell cycle analysis as previously described [31]. In brief, approximately 2×10^5 MCF-7 cells plated in 6-well plates were treated with 5F 203 (0.1, 1.0 or 10.0 µM; 3, 6, 18 or 24 h), harvested in ice-cold PBS (pH 7.4) and fixed in 70% ethanol for at least 30 min. Prior to analysis, cells were washed once with PBS and stained with PBS containing 50 µg/ml propidium iodide and RNase (50 µg/ml) at 37° C for 30 min. Cellular DNA content was analyzed with a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA, USA). Cell cycle distributions were calculated on DNA plots using the Modfit LT Software (Verity Software House, Inc., Topsham, ME, USA).

Topoisomerase I- and topoisomerase II-DNA cleavage assays

Top1 assay

The 161-bp fragment from pBluescript SK (-) phagemid DNA (Stratagene, La Jolla, CA, USA) was cleaved with restriction endonucleases *Pvu*II and *Hind*III (New England Biolabs, Beverly, MA, USA) in supplied NE buffer 2 (50-µl reactions) for 1 h at 37°C as previously described [36]. Briefly, reaction products were separated by electrophoresis in a 1% agarose gel in 1× TBE buffer. The 161-bp fragment was eluted from the gel slice using the QIAEX II kit (QIAGEN Inc., Valencia, CA, USA). The pSK fragment was singly 3′-end-labeled by a fill-in reaction. Linearized pSK (200 ng) was incubated with [α^{32}P]-dGTP in 1× labeling buffer (0.5 mM each dATP, dCTP, dTTP in 50 mM Tris–HCl, pH 8.0, 100 mM $MgCl_2$, 50 mM NaCl) in the presence of 0.5 U of the Klenow fragment of DNA polymerase I. Labeled DNA was purified using mini quick spin DNA columns (Roche Diagnostics Corporation, Indianapolis, IN, USA). Labeled DNA (~50 fmol/reaction) was incubated with 5 ng of recombinant top1 with or without 5F 203 at 25°C in 10 µl reaction buffer (10 mM Tris–Cl pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 µg/ml BSA, final concentrations).

Top2 assay

The pSK fragment (the same as used for top1 reactions) was 5′-end-labeled with [$\gamma-^{32}$P]ATP and T4 kinase. DNA substrates (10 pmol per reaction) were incubated with 500 ng of top2 in the presence or absence of varying concentrations of 5F 203 for 30 min at 25°C in 10 µl of reaction buffer (10 mM Tris–HCl, pH 7.5/50 mM KCl/5 mM $MgCl_2$/1 mM ATP/0.2 mM DTT/0.1 mM EDTA/15 µg/ml BSA) as described previously [16]. VP-16 (100 µM) was used as a positive control.

For both top1 and top2 DNA cleavage assays, reactions were stopped by adding SDS (0.5% final concentration). To the reaction mixtures, 3.3 volumes of Maxam Gilbert loading buffer (80% formamide, 10 mM sodium hydroxide, 1 mM sodium EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue, pH 8.0) were added. Aliquots were separated in 16% denaturing polyacrylamide gels (7 M urea) in 1× TBE (89 mM Tris-borate, 2 mM EDTA, pH 8.0) for 2 h at 40 V/cm at 50°C. Imaging and quantitation were performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

Alamar Blue™ Assay

The cytotoxicity of 5F 203 in XPD and XPD-complemented fibroblasts was determined using the Alamar Blue™ assay as previously described [14]. Cells were plated in 96 well plates at their appropriate densities (1,500–2,500 cells/well) in a total volume of 200 µl. After 2–3 d of incubation, cells were treated with 5F 203 (1 nM–10 µM) for 72 h before the addition of 20 µl of Alamar Blue dye. After 4 h of dye incubation, absorbance was read with a spectrophotometer (530/590 nm). Dose-response curves were plotted to demonstrate sensitivity of these cells to 5F 203. The percentage

of cell growth relative to the untreated control is represented as the mean ± SD of at least three independent experiments.

RNA extraction and quantitative real-time RT-PCR analysis

MCF-7, MDA-MB-435 and XPD cells were plated in T-25 flasks and after reaching logarithmic growth phase, were treated for 24 h with 1 μM 5F 203 or with 0.1% DMSO (vehicle control). The samples were then harvested and RNA extracted according to the manufacturer's instructions (Qiagen, Inc.) Only samples with absorbance values >1.7 for RNA were used in the QRT-PCR. QRT-PCR was performed on these samples to determine the expression of CYP1A1 as previously described [20].

Data analysis

All values are expressed as mean ± SEM. Statistical comparisons between XPD versus XPD-complemented cells treated with 5F 203 were determined using the Student's *t* test. Difference was considered statistically significant indicated by (*) when $P < 0.05$. All statistical analyses were done using Instat software (GraphPad Software, San Diego, CA, USA).

Results

5F 203 arrests cell cycle progression in sensitive MCF-7 cells

Previous studies have shown that 5F 203 selectively inhibits the growth of some cells (i.e., sensitive MCF-7) but not others (i.e., resistant MDA-MB-435) [12, 18, 35]. We used MCF-7 cells which displayed the most remarkable sensitivity, to gain additional insight into the anti-proliferative activity of 5F 203.

In this study, FACS analysis was used to determine whether DNA damaging effects resulting from 5F 203 also had an impact on cell cycle progression. As shown in Fig. 2, MCF-7 cells treated with 5F 203 display a

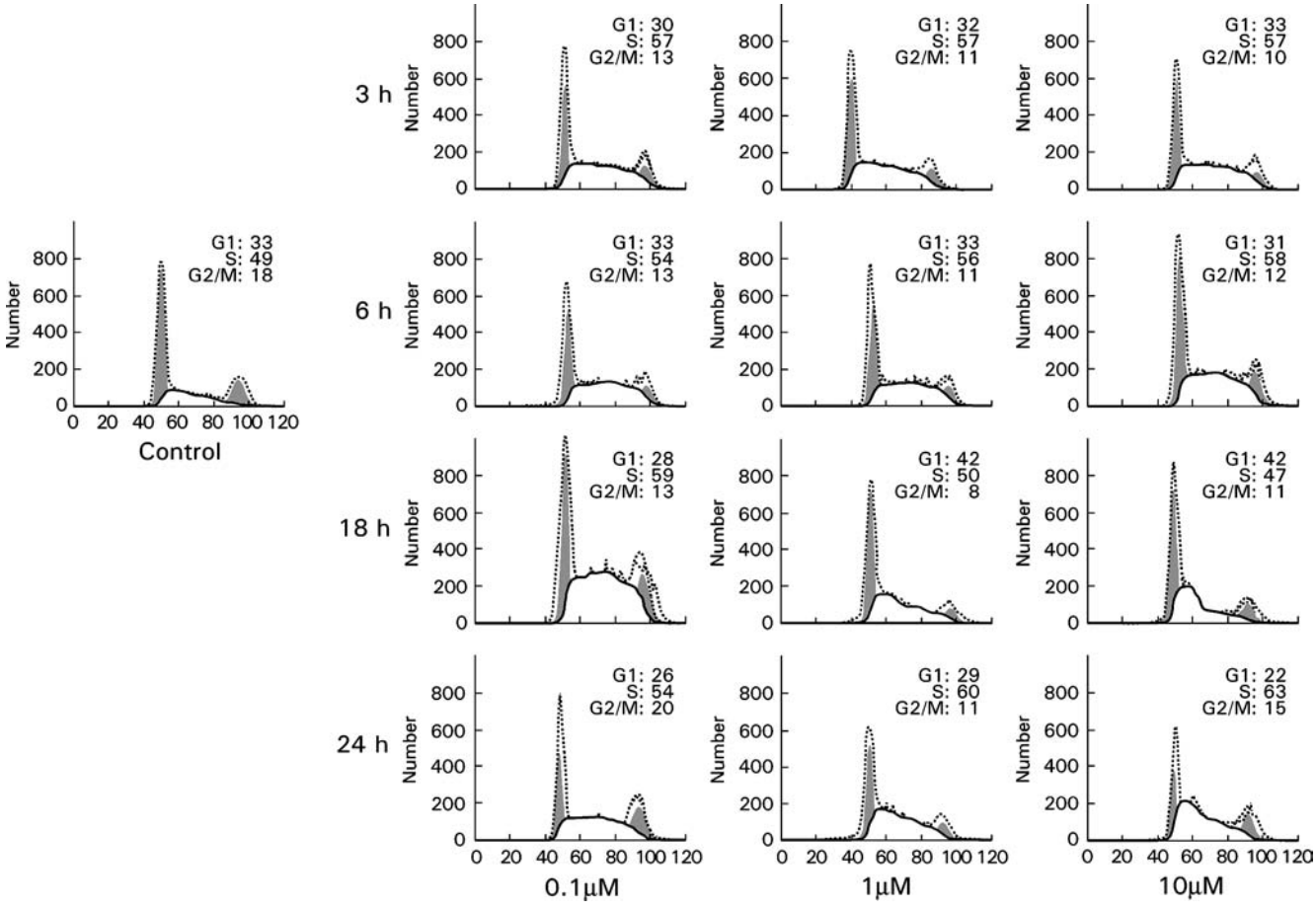

Fig. 2 5F 203 induces cell cycle arrest in MCF-7 cells. Exponentially growing MCF-7 cells were exposed to either 0.1% DMSO (control) or 5F 203 (0.1, 1 or 10 μM) for 3, 6, 18 or 24 h. Cells were then harvested, washed in PBS, and fixed in 70% ethanol. DNA content was evaluated with propidium iodide staining and fluorescence measured and analyzed as described in Materials and Methods. Data are representative of three independent experiments

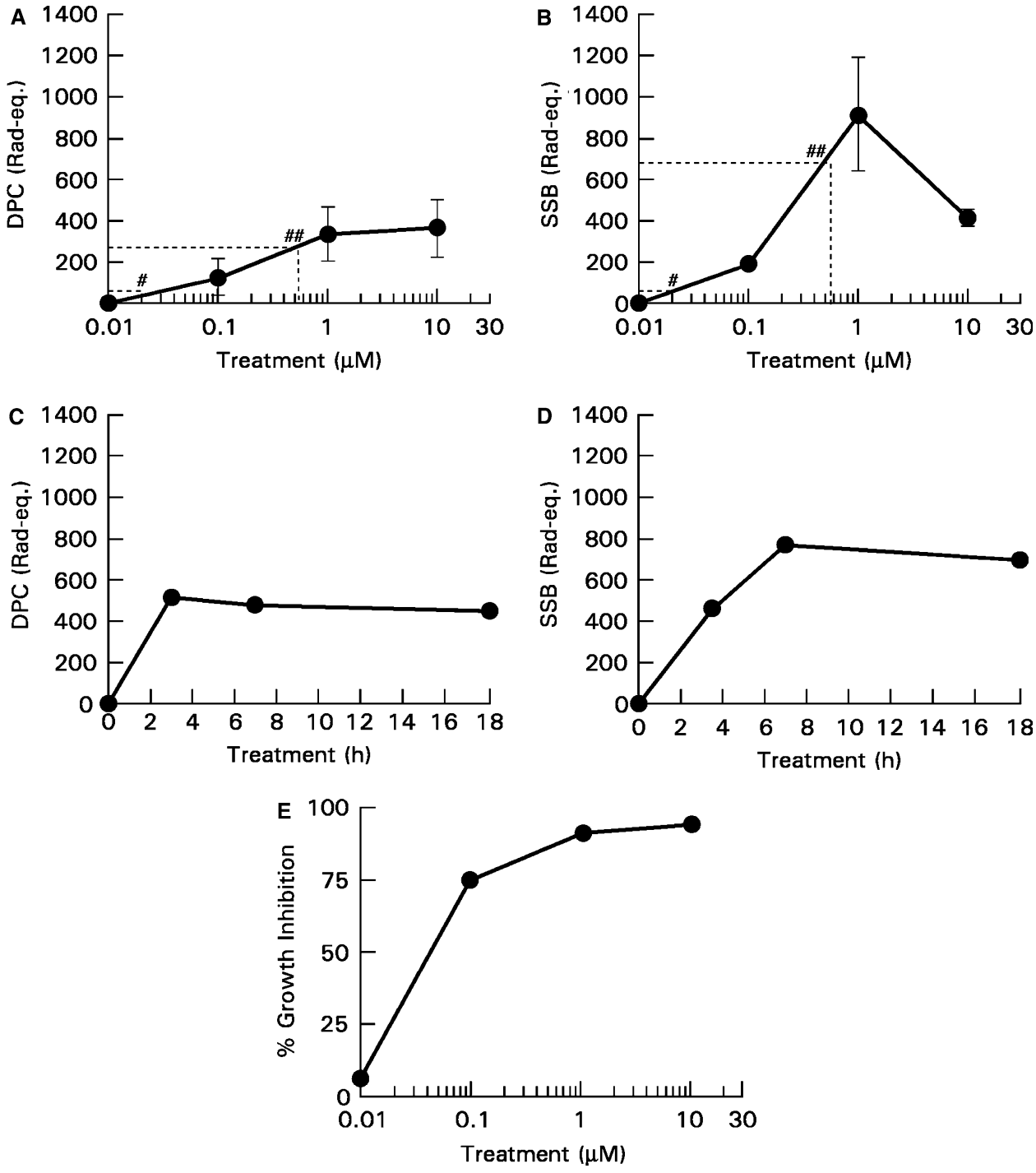

Fig. 3 5F 203 induces dose- and time-dependent single-strand breaks and DNA-protein cross-links in MCF-7 cells. **a**, **b** MCF-7 cells were treated with varying concentrations of 5F 203 for 18 h or with 0.1% DMSO (control) at 37°C before resultant (**a**) DPCs or (**b**) SSBs were assayed by alkaline elution. Data were calculated from the means of three independent experiments performed in duplicate; bars, SD. **c**, **d** MCF-7 cells were treated for 3 h, 7 h and 18 h with 5F 203 (1 μM) or with 0.1% DMSO (control) at 37° C before resulting (**c**) DPCs or (**d**) SSBs were detected using alkaline elution. Data represent two independent experiments performed in duplicate. #Represents the Rad-eq versus [5F 203] required to produce 50% growth inhibition. ##Represents the Rad-eq versus [5F 203] required to produce 100% growth inhibition. **e** MCF-7 cells were plated in 96-well plates and treated with 5F 203 for 6 h (the point at which maximal single-strand breaks and DNA-protein cross-links form) before cell sensitivity was determined using the MTT assay. Each data point is derived from the mean of two independent experiments

pronounced S-phase arrest relative to controls following 3, 6 and 24 h of exposure. We detected G1 arrest following 18 h of 5F 203 exposure with no distinct increase in the accumulation of cells in the S-phase, an affect that was detected following 24 h of 5F 203 exposure in MCF-7 cells in other investigations [19,

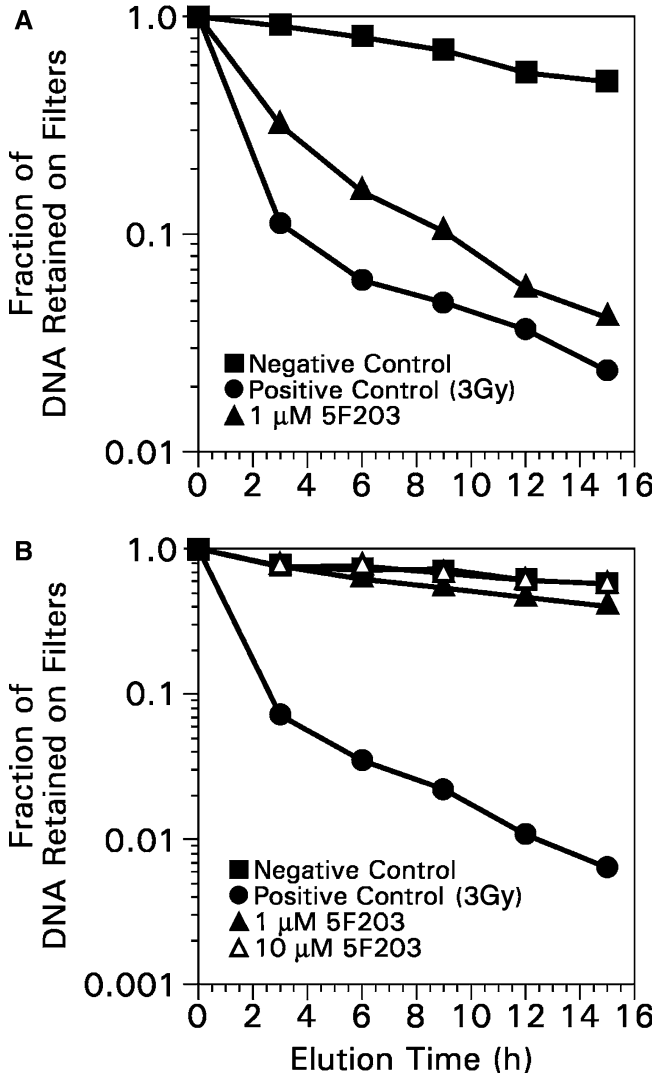

Fig. 4 Single-strand breaks do not readily form in MDA-MB-435 cells. **a**, **b** MCF-7 (**a**) and MDA-MB-435 (**b**) cells were treated for 6 h with 1 μM 5F 203 or with 0.1% DMSO (negative control) at 37°C before being analyzed for single-strand break formation using alkaline elution analysis. Data represent the mean of at least two independent experiments

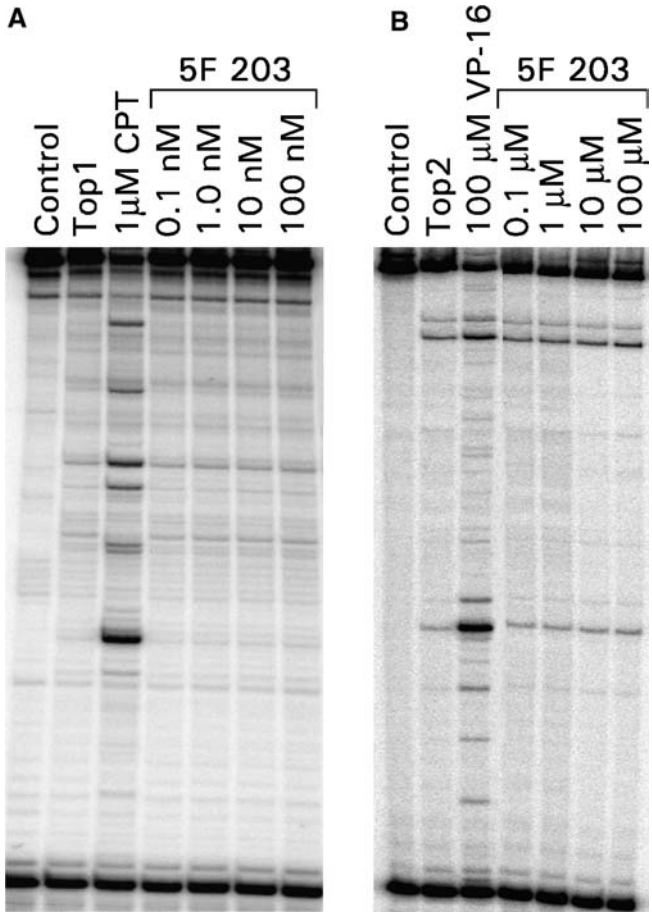

Fig. 5 5F 203 does not trap top1- or top2-DNA cleavage complexes. **a**, **b** ^{32}P-end-labeled *PvuII-HindIII* 161-bp fragment from pBluescript was incubated with purified top1 (**a**) or top2 (**b**) in the presence or absence of CPT or VP-16 respectively or with varying concentrations of 5F 203 at 25°C for 30 min in accordance with *Materials and Methods*. Reactions were stopped with SDS (final concentration, 0.5%) and resolved on 16% denaturing polyacrylamide gels. Imaging and quantitation were performed using a Phosphorimager

35]. Leong et al. [19] demonstrated a decrease in G1 arrest following longer incubations and thus it is possible that our cells mimicked this effect at an earlier exposure time point. Leong et al. [19] also revealed that more pronounced S phase arrest is observed at lower concentrations which is in agreement with what we have demonstrated. The presence of S phase arrest (particularly without G1 arrest) suggests that cells that incur DNA damage are able to continue to proliferate [9]. Since S-phase arrest has been consistently observed in this and prior studies, it is possible that MCF-7 cells respond to 5F 203 treatment under the control of an S-phase checkpoint that regulates cell cycle progression following DNA damage.

5F 203 induces SSBs and DPCs in MCF-7 cells

Cell cycle progression is blocked following DNA damage to enable the cell to repair itself [28]. Since 5F 203 induces cell cycle arrest in MCF-7 cells and is able to form DNA adducts [35], we sought to identify the chemical lesion types that form in these cells (i.e., SSBs, DSBs, DPCs, PASBs, ISCs) by alkaline elution analyses. Figure 3 shows that 5F 203 induced a dose- and time-dependent increase in both SSBs and DPCs reaching a plateau by 7 h (1.0 μM). An increase in the number of SSBs and DPCs occurred with an increase in 5F 203 concentration which correlated with an increase in the percentage of cell growth inhibition (Fig. 3a, b and e; # corresponds with 50% growth inhibition and ## corresponds with 100% growth inhibition). A biphasic dose-response was detected for SSBs such that more SSBs were detected following

1.0 μM 5F 203 treatment as compared with 10.0 μM 5F 203 treatment (Fig. 3b). However, no DSBs, PASBs or ISCs were detected in MCF-7 cells following 7 h, 1.0 μM 5F 203 exposure (data not shown). DSBs in 5F 203-treated MCF-7 cells were detected only following incubations that exceed 48 h [19]. To further confirm the relationship between SSB and cytotoxicity, MCF-7 and MDA-MB-435 cells were treated for 6 h with 5F 203 and SSBs were determined (Fig. 4). The lower the fraction of DNA retained on the filters, the greater the SSB formation. SSBs readily occur in MCF-7 cells after treatment as the fraction retained on the filters more closely resembles that of the positive control (Fig. 4a). On the other hand, in resistant MDA-MB-435 cells, the fraction retained on the filters at either 1 or 10 μM 5F 203 is similar to that of the negative control (Fig. 4b). Thus, while SSBs form in MCF-7 cells following 6 h of 5F 203 treatment, no appreciable SSBs formed in MDA-MB-435 cells. No DPCs formed in MDA-MB-435 cells (data not shown). This further confirms that SSB and DPC formation correlates with cell sensitivity. PASBs were not detected in either of the cell lines, indicating that any 5F 203-induced strand breaks formed were not protein-linked.

5F 203 does not trap top1-DNA cleavage complexes

To further examine whether 5F 203 acts as a direct inhibitor of top1, cell-free in vitro assays that demonstrate a drug's ability to trap and enhance top1-DNA cleavage complexes were conducted. Induction of DNA cleavage complex formation in the presence of top1 was determined using the *Pvu*II/*Hind*III fragment of pBluescript SK(-) phagemid DNA (Fig. 5a) [33]. While CPT was readily able to trap top1-mediated DNA cleavage complexes, increasing concentrations of 5F 203 proved ineffective in trapping top1. This confirms that 5F 203 does not inhibit top1.

5F 203 does not trap top2-DNA cleavage complexes

Topoisomerase II binds DNA, enabling DNA phosphodiester backbone cleavage and the passage of a second DNA duplex; after which the cleaved DNA strand becomes ligated, eventually resulting in enzyme turnover as reviewed in [37]. Anticancer DNA intercalators such as VP-16 inhibit cancer cell growth by trapping DNA cleavage intermediates that are re-ligated, thus generat-

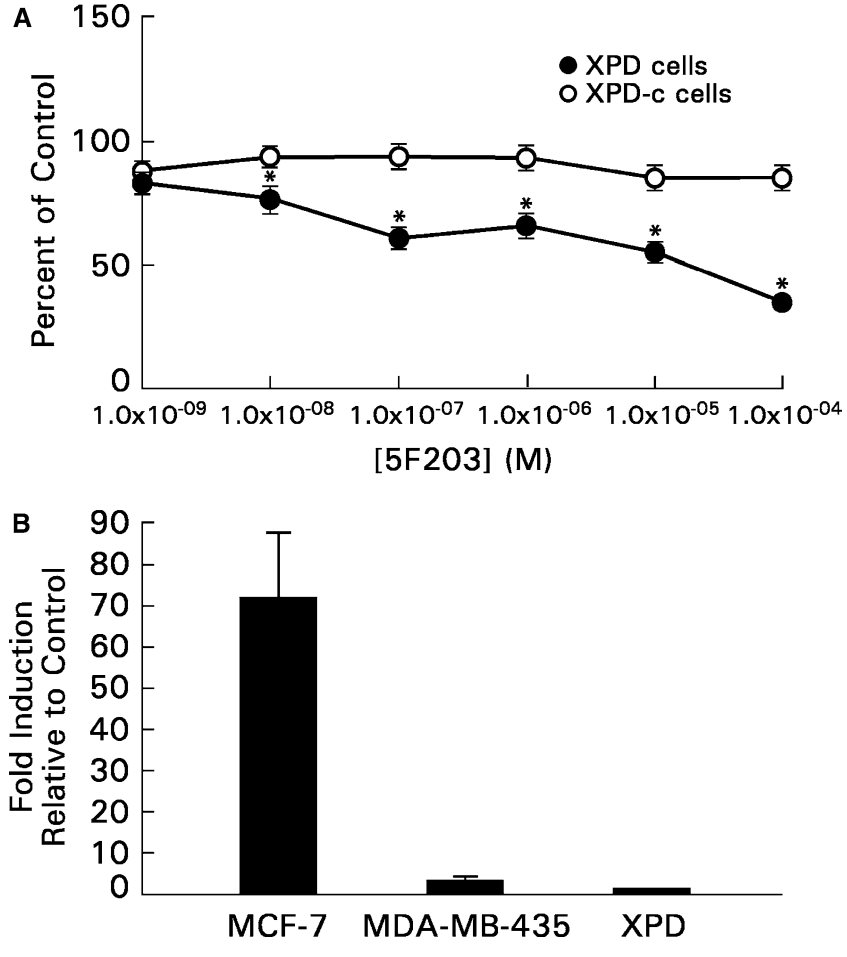

Fig. 6 5F 203 inhibits the growth of XPD but not XPD-complemented (XPD-c) cells. **a** Exponentially growing XPD and XPD-complemented cells were treated with varying concentrations of 5F 203 or vehicle control (0.1% DMSO) continuously for 72 h before Alamar Blue dye reagent was added and fluoresence was measured to determine the anticancer activity of 5F 203 as determined in *Materials and Methods*. Data were calculated as the mean of at least three independent determinations; *bars*, SD. The *asterisk* (*) represents $P < 0.05$, XPD cells versus XPD complemented cells. **b** MCF-7, MDA-MB-435 and XPD cells were treated with 1 μM 5F 203 for 24 h before CYP1A1 mRNA expression levels were determined using QRT-PCR analysis

ing transient double-strand breaks and modifying DNA topology in cancer cells [26].

To determine whether 5F 203 displays anticancer activity by directly inhibiting top2, an in vitro DNA cleavage assay was employed using the same DNA substrate as with top1 except that it was 5′-labeled. Following incubation with human DNA top2 in the presence or absence of 5F 203, top2-DNA cleavage complexes were analyzed as described previously [16]. As anticipated, top2-DNA cleavage complexes were trapped in VP-16-treated DNA substrates as compared to that with top2 alone (control). However, no top2-DNA cleavage complexes were detected in 5F 203-treated samples (Fig. 5b). This indicates that 5F 203 does not target top2.

Xeroderma pigmentosum complement group D-deficient fibroblasts are more sensitive to the anti-proliferative effects of 5F 203 than XPD-complemented fibroblasts, but do not express an increase in CYP1A1 mRNA expression following 5F 203 treatment

5F 203 (1.0 μM) has been shown to form DNA adducts in sensitive MCF-7 cells and the formation of these adducts reaches saturation following 24 h of treatment, potentially due to activation of the nucleotide excision repair (NER) pathway [18]. To determine if the NER pathway may play a role in excising adducts that form in sensitive cells, XPD-deficient fibroblasts and XPD-complemented fibroblasts were treated with varying concentrations of 5F 203 (1 nM–100 μM). Figure 6a shows that XPD-deficient cells showed sensitivity to 5F 203 ($IC_{50} \approx 15$ μM). Alternatively, the corresponding XPD-complemented cells were resistant to 5F 203 treatment ($IC_{50} > 100$ μM).

Previous studies indicate that 5F 203 induces CYP1A1 expression exclusively in sensitive cancer cells [6, 7, 11]. In order to determine if 5F 203 could induce CYP1A1 expression in XPD cells, we performed QRT-PCR to measure CYP1A1 mRNA expression in these cells in comparison to MCF-7 cells and MDA-MB-435 cells. Figure 6b demonstrates that while 5F 203 readily induced CYP1A1 in MCF-7 cells, this benzothiazole was unable to induce CYP1A1 expression in XPD cells and the level of CYP1A1 expression resembled that seen in resistant MDA-MB-435 cells. These data suggest that 5F 203 inhibits the growth of XPD cells via a CYP 1A1-independent mechanism.

Discussion

In this investigation, we demonstrate that 5F 203 induces dose- and time-dependent single-strand breaks and DPCs in sensitive MCF-7 breast cancer cells. We also demonstrate that 5F 203 induces cell cycle arrest following relatively brief exposures (< 24 h) that appears to correlate with the ability of this anticancer drug candidate to induce DNA damage. The anti-proliferative activity of 5F 203 appears to depend on targets other than top1 or top2 and may only be detected in cancer cells lacking effective nucleotide excision repair pathways.

An arrest in cell cycle progression is vital to the maintenance of the genomic stability within cells. An arrest in G1, the ''DNA damage'' checkpoint, creates a pause in the cell cycle that enables the cells to repair DNA damage, while an arrest in the S-phase is critical for preventing the replication of damaged DNA [3]. In this study, the S-phase arrest that was detected following 5F 203 treatment signaled the presence of DNA damage. We detected more prominent S-phase arrest at 10 μM, 24 h 5F 203 exposure and this treatment parameter was shown to produce an additional DNA adduct in sensitive MCF-7 cells in a previous study [18]. Cancer cells treated with 5F 203 have also been shown to undergo apoptosis in response to DNA damage [35], which prompted us to more clearly define the mechanism of anti-proliferative activity for 5F 203.

Because 5F 203 can form DNA adducts [18], we examined the type of DNA lesions induced in MCF-7 cells treated with 5F 203 which would enable us to gain further insight into the mechanism of anti-proliferative activity for 5F 203. The present study shows that 5F 203 induced dose- and time-dependent SSBs and DPCs. Single-strand breaks were more prominent than DPCs in sensitive cells treated with 5F 203. Interestingly, SSBs formed in a biphasic dose-response fashion in 5F 203-treated MCF-7 cells although no distinct biphasic dose-response could be detected in the anticancer drug screen in these cells as demonstrated in a previous study [12]. In general however, we show that cells form DPCs and SSBs following 5F 203 treatment in a manner that correlates with their sensitivity to this anticancer drug candidate (Figs. 3a, b and e, 4a and b). The inability of MDA-MB-435 cells to metabolize 5F 203 and display an induction of CYP 1A1 following 5F 203 treatment also readily distinguishes them from MCF-7 cells as described previously [6]. The exact mechanisms involving the resistance of MDA-MB-435 cells to 5F 203 have not yet been elucidated but we suspect that these cells possess repair mechanisms that prevent the persistant DNA damage needed to demonstrate cytotoxicity.

Following 6 h of 5F 203 treatment, no detectable double-strand breaks nor protein-associated strand breaks, typically associated with topoisomerase I or II inhibition were detected (data not shown). Double-strand breaks only formed in sensitive MCF-7 cells following 5F 203 exposures times exceeding 48 h [19]. Such double-strand breaks could be due to the cells' apoptotic response.

O'Brien et al. [24] have predicted the structural identities of metabolites (presumably electrophilic species) of benzothiazoles quantum mechanically and consider the active intermediate(s) to consist of a nitrenium ion and/or an epoxide that can alkylate DNA. In addi-

tion, O'Brien et al. suggest that these metabolites (intermediate species) bind to DNA to form the vast majority of the resultant adducts responsible for cell death. Since 5-fluoro substitution found in 5F 203 is presumed to prohibit the formation of hydroxylated derivatives, we propose that the predominant metabolites and subsequent DNA adducts that form would consist of derivatives of the nitrenium ion reactive species. Efforts are underway to characterize the DNA adducts that form as single-strand breaks and DNA-protein cross-links and to identify the protein associated with DNA protein-cross-links that form in sensitive cells following 5F 203 treatment.

Camptothecin and etoposide have been shown to induce strand breaks by trapping top1- and top2-DNA cleavage complexes respectively as a means of eliciting their anticancer effects [13, 27]. In this investigation, 5F 203 was unable to trap top1- or top2-DNA cleavages. This strongly suggests that neither topoisomerase I nor topoisomerase II represent critical targets for 5F 203-mediated anti-cancer activity.

Intrinsic or acquired resistance represents a critical limitation in the maintenance of anticancer efficacy in agents such as cisplatin. While these agents may initially form DNA adducts in sensitive cells, resistance often ensues. This increase in resistance has been attributed to diminished drug accumulation, enhanced cellular detoxification as a result of increased levels of glutathione and enhanced DNA repair as reviewed in [5]. Previous studies indicate that cisplatin resistance is primarily related to the ability of cells to repair cisplatin-based adducts via the transcription coupled-nucleotide excision repair (TC-NER) pathway [8].

A previous report indicated that 5F 203 induces the expression of the DNA damage responsive gene, DNA binding protein 2 (DDB2) [23]. Studies suggest an association between the NER pathway and DDB2 [29]. Thus, we suspected that the presence or absence of the XPD gene, a critical gene in the NER pathway, would influence the anti-proliferative activity of 5F 203. DNA adducts are only detectable at concentrations that exceed 0.1 μM suggesting that when lower concentrations of 5F 203 are used, nucleotide excision repair mechanisms may become triggered to eliminate any DNA adducts that initially form [18]. In this study, XPD-deficient cells showed more sensitivity to 5F 203 than the highly resistant XPD-complemented cells.

Figure 6b indicates that 5F 203 was unable to induce the expression of CYP1A1 in XPD-deficient cells similarly to the resistant MDA-MB-435 cells. This indicates that these particular cells may display their sensitivity via a mechanism that does not depend on CYP1A1 induction. It is essential to consider that XPD cells are substantially less sensitive to 5F 203 than MCF-7 cells which may also account for the lack of CYP1A1 induction. Since these cells lack XPD, a critical gene in the nucleotide excision repair pathway, any sensitivity detected in these cells may result from their inability to repair any DNA damage resulting from 5F 203 treatment. Studies are underway to determine whether DNA damage (adducts, SSBs, DPCs) readily occurs and persists in these cells similarly to MCF-7 cells.

Cells defective in specific genes associated with the NER pathway often display enhanced sensitivity to chemotherapeutic agents since inhibition of this pathway results in decreased DNA repair. Inhibiting the NER pathway may be beneficial in overcoming resistance that ultimately undermines chemotherapeutic efficacy [38]. The XPD gene participates in both the transcription-coupled and global-genome NER pathways while the xeroderma pigmentosum complement C (XPC) gene is selectively involved in the global genome NER pathway. We found that XPC-deficient cells were slightly more sensitive to 5F 203 than XPD cells suggesting that the global-genome pathway plays a more active role than the transcription-coupled pathway in the repair of DNA adducts created by 5F 203 (data not shown). However, it is very probable that other repair mechanisms play a more prominent role in DNA adduct removal in sensitive cancer cells since XPD-deficient cells were much less sensitive to the cytotoxic effects of 5F 203 than MCF-7 cells and did not show induced CYP 1A1 expression following treatment (Fig. 6).

Selective uptake, metabolism and induction of DNA damage have been demonstrated as requisite to the anticancer activity of drug candidates such as the ellipticines, thiophene and aminoflavone [1, 17, 30]. Aminoflavone, an anticancer drug candidate that has recently entered phase I clinical trials in the US, is metabolized by CYP1A1, induces CYP1A1/2 expression, cell cycle arrest, apoptosis and DNA adduct formation in sensitive cells [17, 25, 30]. In addition, Aminoflavone also induces DPCs and single-strand breaks and enhances the phosphorylation of γH2AX, p53 and p21 in these cells in a topoisomerase-independent manner [22, 25]. The development of Aminoflavone or 5F 203 as anticancer agents is worthwhile and promising since no drugs currently used in the clinics display a similar mechanism of anticancer activity involving the induction of their own metabolism subsequent to the induction of CYP1A1 and DNA damage. The detection of specific DNA adducts in cancer cells treated with chemotherapeutic agents may prove useful in predicting clinical responsiveness critical in the development of more efficacious therapy.

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